Cancer Therapeutics Insights

Expression of Leukemia-Associated Fusion Proteins Increases Sensitivity to Histone Deacetylase Inhibitor–Induced DNA Damage and Apoptosis

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Abstract

Histone deacetylase inhibitors (HDI) show activity in a broad range of hematologic and solid malignancies, yet the percentage of patients in any given malignancy who experience a meaningful clinical response remains small. In this study, we sought to investigate HDI efficacy in acute myeloid leukemia (AML) cells expressing leukemia-associated fusion proteins (LAFP). HDIs have been shown to induce apoptosis, in part, through accumulation of DNA damage and inhibition of DNA repair. LAFPs have been correlated with a DNA repair–deficient phenotype, which may make them more sensitive to HDI-induced DNA damage. We found that expression of the LAFPs PML-RARα, PLZF-RARα, and RUNX1-ETO (AML1-ETO) increased sensitivity to DNA damage and apoptosis induced by the HDI vorinostat. The increase in apoptosis correlated with an enhanced downregulation of the prosurvival protein BCL2. Vorinostat also induced expression of the cell-cycle regulators p14ARF and p21WAF1 and triggered a G2–M cell cycle arrest to a greater extent in LAFP-expressing cells. The combination of LAFP and vorinostat further led to a greater downregulation of several base excision repair (BER) enzymes. These BER genes represent biomarker candidates for response to HDI-induced DNA damage. Notably, repair of vorinostat-induced DNA double-strand breaks was found to be impaired in PLZF-RARα–expressing cells, suggesting a mechanism by which LAFP expression and HDI treatment cooperate to cause an accumulation of damaged DNA. These data support the continued study of HDI-based treatment regimens in LAFP-positive AMLs. Mol Cancer Ther; 12(8); 1591–604. ©2013 AACR.

Introduction

The pathogenesis of acute myeloid leukemia (AML) involves activating mutation(s) resulting in a proliferative and/or survival advantage and loss of function mutation(s) resulting in a differentiation arrest (1). Leukemia-associated fusion proteins (LAFP), such as PLZF-RARα, PML-RARα, and RUNX1-ETO (referred to as AML1-ETO henceforth), are generated from chromosomal translocations and are involved in blocking differentiation, thus directly driving the malignant phenotype. They do so by recruiting histone deacetylase (HDAC) containing corepressor complexes to the promoters of differentiation genes, resulting in their transcriptional silencing (2, 3). HDACs remove acetyl groups from the lysine residues of histones and other proteins. In the case of histones, deacetylation results in the re-organization of chromatin into a closed conformation that obstructs transcription (4). Targeting the HDAC component of these corepressor complexes with small-molecule HDAC inhibitors (HDI) has proven to be an effective strategy in resensitizing leukemic cells to differentiating stimuli (5, 6). Indeed, preclinical and clinical studies have found HDIs to display activity in a broad range of hematologic and solid malignancies, yet the portion of patients with a given malignancy that experiences a meaningful therapeutic response remains small (4). Thus, HDIs have presently only gained U.S. Food and Drug Administration (FDA) approval for the treatment of cutaneous T-cell lymphoma (7), and it is clear that our understanding of the mechanisms of response to HDIs is still lacking.

In addition to their inhibitory effects on differentiation, multiple groups have reported that expression of LAFPs results in a DNA repair–deficient phenotype (8, 9). Expression of PML-RARα, PLZF-RARα, or AML1-ETO has been shown to downregulate genes implicated in base excision repair (BER), resulting in increased DNA damage (8). Thus, LAFPs may also contribute to the leukemic phenotype by promoting genetic instability and the accumulation of DNA mutations. Importantly, DNA repair capacity has been linked to response to DNA-damaging...
agents (10). Therefore, LAFP-expressing AMLs may represent a group that will respond favorably to DNA-damaging agents. Our laboratory and other groups have shown that one mechanism by which HDIs arrest growth and induce apoptosis in malignant cells is through the accumulation of DNA damage, which can occur through induction of reactive oxygen species (ROS; ref. 11), down-regulation of DNA repair proteins (12–14), replication fork stalling (15), and impairment of DNA repair protein function (12, 16). Interestingly, various HDIs have shown promising activity in LAFP-expressing AMLs. For example, expression of AML1-ETO in U937 cells increased sensitivity to apoptosis induced by the HDI ITF2357 (17). Moreover, in a phase II clinical trial, romidepsin showed greater efficacy in patients with AMLs expressing AML1-ETO and other core binding factors (18). Despite these observations, the role of DNA damage mechanisms remains to be ascertained.

The aim of this study was therefore to investigate the effect of LAFP expression on sensitivity to HDI-induced DNA damage and apoptosis. We have evaluated the impact of HDI treatment on cell lines with inducible expression of PLZF-RARα, PML-RARα, or AML1-ETO. LAFP expression was found to increase sensitivity to HDI-induced DNA damage and apoptosis. LAFP expression and vorinostat treatment alone or in combination were found to significantly downregulate BER genes and correlated with impaired DNA repair. These data show that the DNA damage capabilities of HDI are greater in LAFP-expressing cells and suggest possible biomarkers for response to HDI-induced DNA damage.

Materials and Methods

Reagents and cell lines

Vorinostat was obtained courtesy of Merck & Co. LBH588 and MGCD0103 were obtained courtesy of Novartis AG and MethylGene. Sodium butyrate (NaB) and trichostatin A (TSA) were purchased from Sigma. Romidepsin was purchased from Selleckchem. Chemical structures of HDIs are shown in Fig. 6. NB4, Kasumi-1, and U937 cells were obtained from American Type Culture Collection and cultured in RPMI-1640 (Wisent) supplemented with 10% FBS at 37°C with 5% CO2. U937 is a p53 null histiocytic lymphoma cell line (19). The PLZF-RARβ3 cell line is based on the U937 autoregulatory tetracycline-off system. Cells were cultured as previously described (20), and PLZF-RARα expression was induced by washing cells with PBS and culturing without tetracycline (Sigma). B412, PR9, and U937-A/E cells expressing PLZF-RARα, PML-RARα, or AML1-ETO, respectively, under the control of a Zn-inducible promoter, were obtained from Drs. Martin Ruthardt and Myriam Alcalay (8, 21). SN4 cells (U937 cells stably transfected with empty pSG-MtNeo) were used as a control. B412, PR9, U937-A/E, and SN4 cells were treated with 100 µmol/L ZnSO4 (Sigma) to induce expression of their respective fusion protein. Authentication of NB4, Kasumi-1, PLZF-RARβ3, B412, PR9, and U937-A/E was conducted by assaying for expression of their respective endogenous or inducible LAFPs. Authentication of U937 and SN4 has not been carried out within the last 6 months. NU7026 (Tocris), an ATP-competitive small-molecule inhibitor of DNA-PKcs (22), was dissolved in dimethyl sulfoxide (DMSO). N-Acetyl-l-cysteine (NAC) was purchased from Sigma and dissolved in Dulbeccos’ Modified Eagles’ Media (DMEM; Wisent). Doxorubicin, etoposide, and cisplatin were purchased from Sigma. Z-VAD-FMK was purchased from Promega. Chemical structures for Z-VAD-FMK, NAC, doxorubicin, etoposide, cisplatin, and NU7026 are shown in Fig. 7. Irradiation of cells was done at room temperature using a Theratron T-780 Cobalt Unit located in the Department of Radiation Oncology at the Jewish General Hospital (Montréal, QC, Canada). Radiation was delivered at a rate of 0.66 Gy/min. The cells were returned to an incubator after irradiation and maintained at 37°C with 5% CO2 until further analysis.

Growth assay

PLZF-RARβ3 cells were seeded at 5 × 10⁴ cells/mL in the presence or absence of tetracycline. Viable cells were counted using trypan blue exclusion at days 2 and 5.

Propidium iodide staining

Propidium iodide (PI) staining was done as previously described (11).

Caspase activity assay

Caspase activity was assayed as previously described (11).

Protein quantification

Protein quantification by Western blot analysis was done by first preparing whole-cell extracts from pelleted cells lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0). Western blotting was then conducted to detect protein levels of BCL2, p21WAF1, p16INK4D, Polb2, FEN1, ETO (Santa Cruz Biotechnology); apurinic/apyrimidinic endonuclease 1 (Ape1), γH2AX, p139, pChk2 T68, Chk2 (Cell Signaling); or PML-RARα (Abcam). β-Actin (Sigma) was used to confirm equal protein loading. Protein quantification of pATM S1981 and pDNA-PKcs T2056 (Abcam) was done by flow cytometry. Cells were fixed in 1% paraformaldehyde and then permeabilized by 1% Triton X-100 dissolved in PBS. Permeabilized cells were incubated with primary antibody against pATM S1981 or pDNA-PKcs T2056 at room temperature. Afterwards, cells were washed with PBS and incubated with secondary Alexa Fluor 488 antibody (Invitrogen). Fluorescence was measured by flow cytometry and analyzed using FCS Express v3.0. Ten thousand events were recorded per sample.

COMET assay

Single-cell gel electrophoresis (COMET assay) was conducted under alkaline conditions as previously described.

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(11, 23). COMETs were scored using CometScore (TriTek) for tail moment (TM). TM is presented in arbitrary units (A.U.).

### Fast micromethod

The fast micromethod for single-strand break (SSB) detection was conducted as previously described (24). Briefly, cells were incubated with PicoGreen for 15 minutes and then exposed to alkaline conditions to induce DNA unwinding. PicoGreen preferentially binds to double-stranded DNA but not to single-stranded DNA. The loss of fluorescence caused by DNA unwinding is measured every minute for 30 minutes. DNA strand breaks are expressed as strand scission factor (SSF) multiplied by –1 after 20 minutes of denaturation.

### Quantitative real-time PCR

RNA was isolated from cells using the Absolutely RNA Miniprep Kit (Agilent). cDNA was generated from 1 μg total RNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). mRNA levels for CDKN1A, CDKN1A, APEX1, APEX1, and NBS1, and NBS1 were quantified by measuring the real-time PCR (qRT-PCR) analysis using Power SYBR green hybridization probe and TaqMan Fast Master Mix (Applied Biosystems). mRNA levels for 18S were assessed by qRT-PCR analysis using TaqMan hybridization probe and TaqMan Fast Master Mix (Applied Biosystems). Relative mRNA levels were determined using the ΔΔCt method and 18S served as the endogenous control. qRT-PCR was carried out using the 7500 Fast Real-time PCR System (Applied Biosystems). Primer sets used are presented in Supplementary Fig. S1.

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was conducted as previously described (25). Briefly, PLZF-RARα nonexpressing and expressing PLZFRARβ3 cells were treated with 1.5 μmol/L vorinostat for 6 hours, fixed with 1% formaldehyde and then whole-cell lysates were prepared. Protein lysates (1 mg) were subject to ChIP with histone H3 (acetyl-K9; Millipore), followed by DNA purification and qRT-PCR with the indicated primer sets (Supplementary Fig. S1).

### DNA damage recovery assay

DNA damage was induced in PLZFRARβ3 cells by treating PLZF-RARα-expressing or nonexpressing cells with 1.5 μmol/L vorinostat for 6 hours. Next, vorinostat was washed off using PBS and cells were replated in fresh vorinostat-free media and allowed to recover for 24 hours. Reduction in γH2AX [DNA double-strand break (DSB) marker] was used to assess DNA repair competency. Levels of γH2AX were assayed by Western blotting.

### Statistics

Significance was determined by ANOVA followed by Newman–Keuls posttests using Prism version 4.0 (GraphPad).

### Results

#### Expression of the LAFP PLZF-RARα enhances sensitivity to HDAC inhibitors

To test the effects of LAFP expression on sensitivity to vorinostat-induced cell death, we used PLZFRARβ3 cells, which are U937 cells stably transfected with PLZF-RARα cDNA under the control of a tetracycline-off system (20). When cultured in the absence of tetracycline, PLZFRARβ3 cells express PLZF-RARα (Fig. 1A). Expression of PLZF-RARα did not affect proliferation of PLZFRARβ3 cells within 48 hours of induction. However, the proliferation rate of PLZF-RARα-expressing PLZFRARβ3 cells began to slow down after 48 hours (Supplementary Fig. S2A). Therefore, vorinostat-induced cell death was examined within 48 hours of PLZF-RARα expression. We have previously shown that vorinostat induces significant cell death in U937 cells beginning at 24 hours (11). To assay for cell death, PLZF-RARα-expressing and nonexpressing cells were treated with vorinostat and subsequently stained with PI to quantify fragmented DNA by flow cytometry. Expression of PLZF-RARα caused a striking increase in vorinostat-induced cell death in PLZFRARβ3 cells after 48 hours (Fig. 1B). Tetracycline had no effect on vorinostat-induced cell death in the parental U937 cells (Supplementary Fig. S2B). Similar results were also obtained with B412, a ZnSO4-inducible model for PLZF-RARα expression (ref. 21; Supplementary Fig. S2C). Next, we assessed caspase involvement using a caspase-3/7 activity assay. Expression of PLZF-RARα enhanced vorinostat-induced caspase-3/7 activity (Fig. 1C). An enhanced activation of both caspase-8 (extrinsic apoptosis) and caspase-9 (intrinsic apoptosis) activity was also observed in response to vorinostat in the PLZFRARβ3 expressing cells (Supplementary Fig. S2D). To determine the contribution of caspases, we pretreated PLZF-RARα-expressing and nonexpressing cells with the pan-caspase inhibitor Z-VAD-FMK for 1 hour, followed by vorinostat for 48 hours. Caspase inhibition resulted in decreased vorinostat-induced cell death in both PLZF-RARα-expressing and nonexpressing cells (Fig. 1D), confirming that the cells were dying by apoptosis. The increase in caspase activity led us to investigate the effect of PLZF-RARα and vorinostat on the prosurvival apoptosis regulator BCL2, which was previously shown to be negatively regulated by HDI (26). We confirmed that vorinostat reduced BCL2 mRNA and protein levels and, furthermore, these reductions were enhanced by PLZF-RARα expression (Fig. 1E and Supplementary Fig. S2E). To test whether PLZF-RARα increases sensitivity to HDIs other than vorinostat, PLZF-RARα-expressing and nonexpressing cells were treated with a panel of HDI and assayed for cell death by PI stain. Expression of PLZF-RARα increased sensitivity to LBH589+, TSA+, NaB+, MGCD0103−, and romidepsin-induced cell death (Fig. 1F). Previously, we showed that vorinostat induces ROS in leukemia cells and that pretreatment with the antioxidant NAC could reduce vorinostat-induced cell death.
death (11). Pretreatment with NAC for 1 hour, followed by vorinostat for 48 hours reduced cell death equally in both PLZF-RARα-expressing and nonexpressing cells (Fig. 1G). Consistent with this, vorinostat induced hydrogen peroxide to an equal extent in PLZF-RARα-expressing and nonexpressing cells (Supplementary Fig. S2F), suggesting that enhancement of HDI toxicity by PLZF-RARα is not dependent on ROS.

**Expression of PLZF-RARα enhances vorinostat-induced DNA damage**

We and other groups have shown that vorinostat induces DNA damage (11). LAFPs have also been shown to induce a DNA repair–deficient phenotype (8). Thus, to determine whether PLZF-RARα expression affects vorinostat-induced DNA damage, we conducted single-cell gel electrophoresis (COMET assay) on PLZFRARβ3 cells.
The COMET assay was conducted under alkaline conditions, allowing detection of DNA SSBs, DNA DSBs, and alkali-labile sites. As previously observed, expression of PLZF-RARα resulted in an increased COMET tail moment (8). Moreover, treatment with vorinostat resulted in a greater COMET tail moment in PLZF-RARα-expressing cells than in nonexpressing cells (Fig. 2A). Doxorubicin was used as a positive control and also induced a greater tail moment in PLZF-RARα-expressing cells (Fig. 2A). Next, we conducted the fast micromethod to specifically assay for SSBs (24). This confirmed that PLZF-RARα expression and vorinostat treatment alone induced SSBs (Fig. 2B), whereas PLZF-RARα expression combined with vorinostat treatment resulted in the greatest induction of SSBs (Fig. 2B). Phosphorylation of H2AX (γH2AX) localizes to DSBs within minutes of their formation, making it a sensitive marker for this lesion (27). We observed no increased γH2AX in untreated cells expressing PLZF-RARα, but the combination of PLZF-RARα expression and vorinostat induced γH2AX to a greater extent than vorinostat alone (Fig. 2C). Supporting our findings, an increased TM and γH2AX induction by vorinostat in cell expressing PLZF-RARα was also observed in B412 cells (Supplementary Fig. S3A and S3B). We also tested the ability of the only other FDA-approved HDI, romidepsin, to induce γH2AX in PLZF-RARα3 cells expressing PLZF-RARα or not. Interestingly, romidepsin was observed to induce γH2AX to the same extent in both PLZF-RARα-expressing and nonexpressing cells (Fig. 2C), despite the fact that cell death was enhanced (Fig. 1F).

Induction of DNA damage in leukemic stem cells expressing LAFP, or following HDI treatment, has been described to correlate with induction of the cell-cycle regulator CDKN1A (p21WAF1), refs. (28–30). HDI and DNA damage have also been shown to induce the cell-cycle regulator CDKN2D (p16INK4D); refs. 31, 32). Consistent with these findings, we found that both PLZF-RARα and vorinostat induced p21WAF1 and p16INK4D at the mRNA (Fig. 2D) and protein level (Fig. 2E and Supplementary Fig. S3C). We found that the combination of PLZF-RARα and vorinostat resulted in a greater induction of both p21WAF1 and p16INK4D. While the increase in p16INK4D was transient, p21WAF1 remained high for at least 24 hours (Fig. 2D and 2E). We also assessed expression of the cell-cycle regulator CDKN1B (p27KIP1), which has previously been shown to be induced in response to PLZF-RARα expression (20). We confirmed that PLZF-RARα increases CDKN1B mRNA levels, but vorinostat was observed to downregulate CDKN1B (Supplementary Fig. S3D).

To determine whether the enhanced induction of p21WAF1 correlated with increased histone acetylation, we conducted ChIP analysis using an antibody specific to histone H3 (acetyl-K9). As expected, vorinostat caused a substantial increase in histone H3 (acetyl-K9) enrichment at the p21WAF1 gene; however, expression of PLZF-RARα did not enhance this effect (Supplementary Fig. S3E).

To assess whether PLZF-RARα expression could increase sensitivity to other DNA damaging stimuli, we tested PLZF-RARα-expressing and nonexpressing cells against a panel of such stimuli. Indeed, PLZF-RARα increased sensitivity to cell death induced by γ-irradiation (IR) and the cytotoxic agents doxorubicin, etoposide, and cisplatin (Fig. 2F).

Expression of PLZF-RARα enhances the DNA damage checkpoint response to vorinostat

DNA damage results in the phosphorylation and activation of phosphoinositide 3-kinase–related kinases like ATM and DNA-PKcs. These kinases function to detect DNA damage and signal for a cell-cycle arrest to allow for proper repair of DNA lesions (33). We used flow cytometry to measure phosphorylation of ATM at serine 1981 (pATM S1981) and DNA-PKcs at threonine 2056 (pDNA-PKcs T2056). We found that although expression of PLZF-RARα did not induce phosphorylation of either ATM or DNA-PKcs, it enhanced vorinostat-induced phosphorylation of both proteins (Fig. 3A). Activated ATM and DNA-PKcs phosphorylate and activate downstream checkpoint proteins, including Chk2 (34, 35). Thus, to validate ATM and DNA-PKcs activation, we assayed for Chk2 phosphorylation at threonine 68 (pChk2 T68) by Western blotting. Vorinostat alone can induce Chk2 phosphorylation, however, expression of PLZF-RARα enhances this induction (Fig. 3B). In addition, pretreatment of cells with NU7026, a DNA-PKcs small-molecule inhibitor (22), greatly reduced Chk2 phosphorylation (Fig. 3B). To determine whether activation of checkpoint kinases correlated with a cell-cycle arrest, we stained cells with PI and measured their DNA content by flow cytometry. Vorinostat induced a G2/M arrest and this was enhanced by PLZF-RARα expression (Fig. 3C). While pretreatment with NU7026 did not affect the cell-cycle arrest induced by vorinostat alone, it greatly reduced the percentage of cells arresting in the G2/M cell-cycle phase in response to vorinostat combined with PLZF-RARα expression (Fig. 3C).

Of note, romidepsin also induced a G2/M cell-cycle arrest in PLZF-RARβ3 cells, but expression of PLZF-RARα did not augment this arrest (Supplementary Fig. S4). This is consistent with the fact that PLZF-RARα did not increase romidepsin-induced γH2AX (Fig. 2C).

Other LAFPs also increase cell death and DNA damage in response to vorinostat

Vorinostat induces significant cell death in cell lines that endogenously express LAFPs like NB4 (PML-RARα) and Kasumi-1 (AML1-ETO; Supplementary Fig. S5A). To further investigate whether these LAFPs can increase sensitivity to vorinostat, similar to PLZF-RARα, we used U937 clones that conditionally express PML-RARα (PR9) or AML1-ETO(U937-A/E). These cells are stably transfected with PML-RARα or AML1-ETO cDNA under transcriptional control of a Zn-inducible mouse metallothionein promoter (ref. 8; Supplementary Fig. S5B). A U937 clone...
Figure 2. Expression of PLZF-RARα enhances vorinostat-induced DNA damage. PLZF-RARα–nonexpressing and -expressing PLZFRARβ3 cells were treated with 1.5 μmol/L vorinostat or 5 nmol/L romidepsin for the indicated time points. A, cells were assayed for DNA SSBs, DSBs, and alkali-labile sites by alkaline COMET assay. Doxorubicin was used as a positive control. At least 100 nuclei were randomly selected and quantified for DNA damage, represented as an increase in TM (A.U.). Representative images of COMET tails obtained are presented in the panel to the right. B, cells were assayed for SSBs by the fast micromethod. The extent of DNA strand breaks is expressed as strand scission factor multiplied by -1 (SFF x -1). C, induction of the DNA DSB marker γH2AX was measured by Western blotting. β-Actin was used as a loading control. D, PLZF-RARα–nonexpressing and -expressing cells were treated with 1.5 μmol/L vorinostat for 3, 6, or 18 hours. Relative mRNA levels for CDKN1A (p21WAF1) and CDKN2D (p19INK4D) were assayed by qRT-PCR. E, PLZF-RARα–nonexpressing and -expressing PLZFRARβ3 cells were treated with 1.5 μmol/L vorinostat for 3, 6, 18, or 24 hours. Protein levels for p21WAF1 and p19INK4D were analyzed by Western blotting. β-Actin was used as a loading control. F, PLZF-RARα–nonexpressing and -expressing PLZFRARβ3 cells were treated with 0.25 μmol/L doxorubicin, 0.5 μmol/L etoposide, 10 μmol/L cisplatin, or 20 Gy IR for 48 hours. Cells were stained with PI and analyzed by flow cytometry for cell death. Asterisks indicate a significant difference (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
(SN4) containing the empty cloning vector was used as a control. Cell death was assayed by PI stain after LAFP-expressing and nonexpressing cells were treated with vorinostat. Expression of PML-RARα and AML1-ETO had an effect on vorinostat-induced DNA damage, we conducted an alkaline COMET assay. As with PLZF-RARα expression, induction of PML-RARα or AML1-ETO increased vorinostat-induced DNA damage (Fig. 4B). Doxorubicin was used as a positive control and also induced a greater TM in LAFP-expressing cells (Fig. 4B). The effect of PML-RARα and AML1-ETO on vorinostat-induced DNA damage was then assayed by γH2AX Western blotting. Expression of PML-RARα, AML1-ETO, or vorinostat treatment alone induced γH2AX, and the combination of PML-RARα or AML1-ETO expression and vorinostat treatment resulted in a greater induction of γH2AX (Fig. 4C).

Vorinostat and LAFPs downregulate DNA repair proteins and inhibit repair

A previous study established LAFP expression downregulates mRNA levels of BER genes and inhibits DNA repair (8). To determine the combined effect of LAFP expression and vorinostat on repair gene expression, we conducted qRT-PCR for PLZFRARβ3 (PLZF-RARα–inducible) and U937-A/E (AML1-ETO–inducible) cells treated with 1.5 μmol/L vorinostat for 18 hours. As previously reported, expression of LAFP resulted in decreased mRNA levels for the BER genes APEX1, POLD2, POLD3, and FEN1 (Fig. 5A and B). We found OGG1 mRNA levels to be reduced with AML1-ETO but slightly increased by PLZF-RARα expression (Fig. 5A and B). Vorinostat treatment alone reduced mRNA levels of all BER genes in both cell line models (Fig. 5A and B). While the combination of PLZF-RARα and vorinostat resulted in a further decrease in BER gene mRNA levels (with the exception of OGG1), this was not observed when combining AML1-ETO expression and vorinostat (Fig. 5A and B). To better understand the mechanism of repression of the BER genes by HDI and LAFP, we assessed the level of histone H3 (acetyl-K9) associated with the APEX1 gene. Interestingly, vorinostat mediated downregulation of APEX1 mRNA correlated with an overall enrichment of histone H3 (acetyl-K9) at all sites of the APEX1 gene assessed, except near the transcriptional start site (~78 kb). Expression of PLZF-RARα did not significantly alter
the level of histone H3 (acetyl-K9) at this gene (Supplementary Fig. S6A). We further conducted Western blot analysis to validate the changes in BER enzyme mRNA levels observed at the protein level. Expression of PLZF-RARα alone reduced protein levels of Ape1 (APEX1) and FEN1 but had no effect on Pol δ2 (POLD2). The combination of PLZF-RARα and vorinostat enhanced the down-regulation of Ape1 and Pol δ2 but did not further reduce FEN1 protein levels (Fig. 5C). Expression of AML1-ETO alone reduced Ape1 protein levels but had little effect on Pol δ2 or FEN1. Vorinostat treatment alone also induced a downregulation of Ape1 and FEN1 protein, whereas little change in Pol δ2 was observed (Fig. 5C).

As HDI have also been shown to downregulate DSB repair genes and vorinostat induced γH2AX, a marker of DSB, to a greater extent in LAFP-expressing cells (Supplementary Fig. S6A). We further conducted Western blot analysis to validate the changes in BER enzyme mRNA levels observed at the protein level. Expression of PLZF-RARα alone reduced protein levels of Ape1 (APEX1) and FEN1 but had no effect on Pol δ2 (POLD2). The combination of PLZF-RARα and vorinostat enhanced the down-regulation of Ape1 and Pol δ2 but did not further reduce FEN1 protein levels (Fig. 5C). Expression of AML1-ETO alone reduced Ape1 protein levels but had little effect on Pol δ2 or FEN1. Vorinostat treatment alone also induced a downregulation of Ape1 and FEN1 protein whereas little change in Pol δ2 was observed (Fig. 5C).

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To assess functional DNA repair capacity, PLZF-RARα nonexpressing and expressing cells were treated with 1.5 μmol/L vorinostat for 6 hours to induce DNA DSBs. Next, cells were washed, replated in vorinostat-free media, and given 24 hours to recover. Changes in γH2AX levels were evaluated by Western blotting to measure DNA repair. After 6 hours with vorinostat, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11). Importantly, in cells lacking PLZF-RARα, γH2AX was increased, consistent with previous data (11).

To ensure changes in γH2AX levels were not due to changes in cell death, we conducted PI stain on cells taken from each condition in parallel. We observed no cell death in any of the conditions (data not shown).

Discussion

LAFPs have been shown to downregulate DNA repair genes resulting in a DNA repair–deficient phenotype (8). HDIs have been shown to increase levels of DNA damage
by numerous mechanisms (11–15). In this work, we examined whether expression of LAFPs would improve response to HDI treatment. We show for the first time that expression of the LAFPs PLZF-RARα, PML-RARα, and AML1-ETO increases HDI-induced DNA damage (Fig. 2 and 4) and apoptosis (Fig. 1). We show that

Figure 5. Vorinostat and LAFPs downregulate DNA repair proteins and inhibit repair. RNA was isolated from cells and converted into cDNA to quantify relative mRNA levels of the BER genes: APEX1 (Ape1), POLD2 (Pol δ2), POLD3, FEN1 and OGG1 by qRT-PCR. Relative mRNA levels ($\Delta\Delta C_T$) of BER genes were measured in (A) PLZFRARα3 (PLZF-RARα-inducible) and (B) U937-A/E (AML1-ETO-inducible) cells treated with 1.5 μmol/L vorinostat for 18 hours. C, changes in BER gene mRNA levels were validated in PLZFRARα3 and U937-A/E cells at the protein level by Western blotting. β-Actin was used as a loading control. D, DNA repair was assessed using a DNA damage recovery assay in PLZFRARα3 cells. Cells were treated with 1.5 μmol/L vorinostat for 6 hours to induce DNA DSBs. Vorinostat was then washed off, and cells were replated in vorinostat-free media and allowed to recover for 24 hours. Western blotting was used to evaluate any reductions in the DNA DSB marker γH2AX, which reflects the amount of DNA repair. β-Actin was used as a loading control. Asterisks indicate a significant difference (\*P < 0.01; \**P < 0.001). Decimals indicate a significant difference versus vehicle (no LAFP)-treated control (***P < 0.001).
increased levels of DNA damage correlate with a down-regulation of DNA repair proteins by LAFPs and/or vorinostat and reduced DNA repair capacity (Fig. 5). Interestingly, we found differences between the effects of vorinostat and romidepsin. While romidepsin also induced greater cell death in PLZF-RARα-expressing cells (Fig. 1F), this did not correlate with an enhanced cell-cycle arrest (Supplementary Fig. S4) or increased levels of DNA damage (Fig. 2C). We found that romidepsin downregulated BCL2 protein to a greater extent in PLZF-RARα-expressing cells (Supplementary Fig. S7A). This suggests that while the enhanced cell death induced by romidepsin is independent of DNA damage, it may still be mediated by an enhanced effect on apoptotic mediators.

DNA damage is often accompanied by an induction of cell-cycle inhibitor proteins and a cell-cycle arrest to allow for proper DNA repair (36). Consistently, we observed an enhanced G2–M arrest (Fig. 3C). The additional DNA damage induced by vorinostat in PLZF-RARα cells correlated with activation of DNA-PKcs and a greater G2–M arrest. While pretreatment with KU7026 blocked Chk2 phosphorylation, it did not completely block the vorinostat-induced G2–M arrest. This suggests that activated pATM S1981 may compensate by activating a different downstream cell-cycle arrest mediators such as Chk1. We also observed an enhanced induction of the cell-cycle inhibitor proteins p21WAF1 and p19INK4D in PLZF-RARα-positive cells treated with vorinostat (Fig. 2D and E). Of interest, Pellici’s group recently showed that expression of LAFPs in mouse hematopoietic stem cells (HSC) induces DNA damage and p21WAF1 (28). Activated p21WAF1 arrested the cell cycle, allowing for DNA repair and protection of the leukemic HSCs. This raises the concern that in an in vivo or HSC setting, despite the potential for vorinostat to induce greater amounts of DNA damage (and apoptosis) in LAFP-expressing cells, leukemic stem cells may remain protected due to increased p21WAF1 activation. The development of drugs or strategies to target p21WAF1 or p21WAF1-mediated cell-cycle arrest may offer an approach to further improve HDI efficacy in LAFP-positive AML cells and LAFP-positive leukemic HSCs. Indeed, a recent study by Bug’s group investigating the effects of HDI on leukemic HSCs supports this concern (37). They found that HDI treatment of PLZF-RARα- or AML1-ETO-expressing murine leukemic HSCs impaired their self-renewal potential as assayed by serial replating experiments. However, residual colony formation was still observed at later replatings in PLZF-RARα-expressing leukemic HSCs treated with vorinostat (37).

It is noteworthy that the enhanced upregulation of p19INK4D in PLZF-RARα-expressing cells treated with vorinostat dissipates over time and that vorinostat
downregulates PLZF-RARα induced p27KIP1 (Fig. 2D and E and Supplementary Fig. S3D). Previous studies have shown p19INK4D and p27KIP1 to induce a G1 arrest, thereby protecting cells from DNA damage (31, 38). In our model, vorinostat induced a G2–M arrest (Fig. 3), suggesting p19INK4D is insufficient to mount a G1 arrest or that the G2–M arrest response is stronger. Interestingly, vorinostat eventually abrogates p19INK4D protein expression compared with vehicle-treated cells expressing PLZF-RARα (Fig. 2E). Given the findings of Pelicci’s group (28), the induction of a cell-cycle regulator is not a desirable event in the context of leukemogenesis or leukemic HSC clearance. Therefore, it is tempting to speculate that the eventual suppression of PLZF-RARα-induced p19INK4D and p27KIP1 may represent a mechanism by which vorinostat inhibits a cell-cycle regulator that could potentially protect leukemic HSCs.

The DNA damage we observed is dependent on 2 factors: DNA damage induced by LAFPs and/or vorinostat and reduced DNA repair capacity. Our group and others have previously shown that vorinostat may induce DNA damage through ROS production (11, 14). Several studies have also shown HDI to have radiosensitizing effects in a variety of cancer models (13, 39). However, PLZF-RARα expression did not lead to increased ROS production in response to vorinostat in our cells, suggesting the enhanced DNA damage is independent of ROS. Expression of LAFPs was previously shown to downregulate a number of BER genes (8). HDIs have been shown to downregulate a number of DNA repair enzymes including enzymes implicated in BER and DSB repair.
Therefore, we sought to investigate the effect of vorinostat on these same genes in the presence or absence of PLZF-RARα or AML1-ETO. Interestingly, vorinostat alone was found to downregulate all BER and DSB repair genes tested at the mRNA level (Fig. 5A and B and Supplementary Fig. S6C). In PLZFRARβ3 cells, the combination of PLZF-RARα and vorinostat treatment resulted in a greater decrease in BER gene mRNA levels than either alone (Fig. 5A). In U937-A/E cells, however, the combination of AML1-ETO expression and vorinostat only caused a greater reduction in Pol β mRNA levels (Fig. 5B). The effects on BER enzyme protein levels were not as dramatic as observed at the mRNA level and were observed only at later time points (18–24 hours, Fig. 5A–C). The fact that the accumulation of DNA damage is observed earlier (12 h, Fig. 2B) thus suggests the presence of additional mechanisms of either enhanced DNA damage or DNA repair suppression. However, the later downregulation of BER enzymes is still significant as it likely contributes to the continued or accelerated accumulation of DNA damage. Moreover, while we observed overlap in the DNA repair genes downregulated by LAFPs and vorinostat, the literature suggests that there are possibly other repair genes and pathways uniquely regulated by either as well (12, 14, 15, 28). Indeed, we found that several DSB repair genes were downregulated by HDI, LAFP, or both (Supplementary Fig. S6B and S6C). This may help explain why the greatest amount of DNA damage is observed when LAFP expression is combined with vorinostat treatment (Fig. 2).

Of note, Ape1 protein levels were strongly reduced in both PLZF-RARα- and AML1-ETO-expressing cells compared with nonexpressing cells (Fig. 5C). The combination of vorinostat treatment and expression of either PLZF-RARα or AML1-ETO expression also resulted in a greater reduction of Ape1 protein levels (Fig. 5C). Ape1 is critical to the BER pathway, implicated in the repair of 95% of all apurinic/apyrimidinic sites (42). Ape1 also possesses a redox domain, which allows it to alter transcription factor DNA binding and therefore gene expression. Through its redox function, Ape1 has been shown to regulate the p53, AP-1, and HIF-1α transcription factors, and expression of their downstream target genes implicated in homologous recombination repair, mismatch repair, and global genome repair (43). Accordingly, Ape1-knockout mice die early in embryonic development (44), whereas heterozygous Ape1 mice experience increased spontaneous mutagenesis (45). This suggests that Ape1 downregulation may be integral to the enhanced DNA damage observed in LAFP-expressing cells treated with vorinostat. Generally, Ape1 overexpression has been correlated with aggressive proliferation and poor prognosis (43, 46, 47). Decreasing Ape1 levels via knockdown or chemical inhibition has been shown to reduce cancer cell growth and sensitize cells to DNA-damaging agents (48, 49). We also found that vorinostat alone downregulates Ape1 in Kasumi-1 cells (Supplementary Fig. S7B). This suggests that vorinostat or other HDI may represent a novel strategy to target Ape1. Alternatively, knocking-up Ape1 may reduce HDI efficacy thereby validating Ape1 as an HDI target and marker of HDI response or resistance. Our data also provide a rationale to future testing of HDI in combination with Ape1 inhibitors.

In the clinic, HDIs have shown promising results in LAFP-positive patients with AMLs. A small phase II trial by Stock’s group showed that HDI had improved anti-leukemic activity in patients with AMLs with LAFPs (18). Twenty patients were separated into 2 groups on the basis of the absence or presence of LAFPs and treated with the HDI romidepsin. While there was no response to HDI by the LAFP-negative cohort, HDI displayed antileukemic activity in 3 of 5 patients from the LAFP-positive cohort. However, disease progression eventually occurred in all patients. Our findings suggest that response may have been linked to romidepsin-induced DNA damage and regulation of apoptosis and DNA repair genes. For future trials, evaluation of DNA damage and DNA repair gene expression may lead to predictive biomarkers for the response to HDI in LAFP-positive AMLs.

In summary, we present evidence that HDI display enhanced activity in LAFP-expressing AML cells. We show that vorinostat treatment leads to a greater accumulation of DNA damage in LAFP-expressing cells and correlates with a downregulation of DNA repair enzymes and impaired DNA repair. Presently, HDIs have not been approved for the treatment of AMLs, as results from the clinic have been mixed (18, 50, 51). Our data show that HDI treatment may be more relevant in LAFP-positive patients and support the continued clinical evaluation of HDI in this setting.

Disclosure of Potential Conflicts of Interest

J.D. Licht has commercial research support from Epizyme, Inc. No potential conflicts of interest were disclosed by the other authors.

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AML Fusion Proteins Enhance DNA Damage by HDAC Inhibitors

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Expression of Leukemia-Associated Fusion Proteins Increases Sensitivity to Histone Deacetylase Inhibitor–Induced DNA Damage and Apoptosis

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